Refinement of the *Xanthomonas campestris* pv. *vesicatoria* *hrpD* and *hrpE* Operon Structure

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The plant-pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* possesses a type III secretion (T3S) system which is encoded in the 23-kb hypersensitive response and pathogenicity (*hrp*) gene cluster. The T3S system is essential for pathogenicity in susceptible hosts and the induction of the hypersensitive response in resistant plants. In this study, we revisited the operon structure of the right part of the *hrp* gene cluster. Based on complementation experiments of transposon insertions and reverse-transcription polymerase chain reaction analyses, the *hrpD* operon contains *hrcQ*, *hrcR*, *hrcS*, and *hpaA*, whereas *hrcD*, *hrpD6*, and *hrpE* belong to the *hrpE* operon. We determined the transcriptional start site of the *hrpE* operon and showed that there is a promoter upstream of *hrcD* containing a plant-inducible promoter box. Conserved secondary mRNA structures in the intergenic region between *hrpD6* and *hrpE* suggest a posttranscriptional regulated expression of *hrpE*. Based on comparisons of different *hrp* gene clusters and the analysis of evolutionary rates, we propose that the *hrpE* transcriptional unit was integrated into the *hrp* gene cluster at a later time.

The bacterial genus *Xanthomonas* includes a wide range of important plant-pathogenic species infecting more than 120 monocotyledonous and 260 dicotyledonous plant species. The different pathovars elicit a wide range of disease symptoms, such as leaf spots and necrosis in host plants, and a hypersensitive response (HR) in resistant plants (Chan and Goodwin 1999). The HR is a rapid, local, programmed cell death that is induced upon recognition of the pathogen and is concomitant with the inhibition of pathogen growth within the infected plant tissue (Klement 1982).

Xanthomonas campestris pv. *vesicatoria*, the causal agent of bacterial spot disease in pepper and tomato plants, is one of the model organisms for the study of plant–pathogen interactions. The bacterial ability to grow and to cause disease on host plants, as well as to elicit the HR in resistant hosts depends on the hypersensitive response and pathogenicity (*hrp*) genes which are located in a contiguous 23-kb *hrp* gene cluster (Bonas et al. 1991). *hrp* gene clusters have been identified in most gram-negative plant-pathogenic bacteria, including *Erwinia chrysanthemi*, *Ralstonia solanacearum*, *Acidovorax ave-*

nae subsp. *citrulli* (AC AY898625), and different pathovars of *Pseudomonas syringae* and *Xanthomonas* spp. (Bauer et al. 1994; Boucher et al. 1987; Frederick et al. 2001; Gürlebeck et al. 2006; Lindgren et al. 1986). Based on common regulatory components and their genetic organization, the *hrp* gene clusters were classified into two main groups. *Erwinia* spp., *Pantoea stewartii* subsp. *stewartii*, and *Pseudomonas syringae* form group I, while *Xanthomonas* spp., *A. avenae*, and *R. solanacearum* belong to group II (Alfano and Collmer 1997). The classification does not reflect the evolutionary relationship of the species and suggests that the *hrp* gene cluster has been transferred horizontally among phylogenetically unrelated bacteria (Gophna et al. 2003).

The *hrp* gene cluster of *X. campestris* pv. *vesicatoria* contains at least six transcriptional units, *hrpA* to *hrpF* (Bonas et al. 1991). The majority of the gene products in the *hrp* gene cluster are involved in the assembly of a type III secretion (T3S) system. The T3S system is a specialized secretion system that transports proteins across the two bacterial membranes and the host cell plasma membrane into the host cell cytoplasm. Eleven genes of the *hrp* gene cluster are conserved among plant and animal pathogens and, therefore, have been termed *hrp*-conserved (*hrc*) genes (Bogdanove et al. 1996; Weber et al. 2005). Eight *hrc* gene products are associated with bacterial membranes and build up a trans-envelope multiprotein complex (He et al. 2004). The remaining three Hrc proteins, predicted to be localized in the bacterial cytoplasm, probably are involved in energizing the transport process (Gonzalez-Pedrajo et al. 2002; Pozidis et al. 2003). The *hrc* gene products are encoded by the *hrpA*, *hrpB*, *hrpC*, and *hrpD* operons. In contrast, the *hrpE* and *hrpF* transcriptional units encode *hrp* and *hrp*-associated (*hpa*) genes, which are found only in other *Xanthomonas* strains. HrpE is encoded in the *hrpE* operon and is the major subunit of a filamentous structure, the Hrp pilus. The Hrp pilus extends up to several micrometers from the bacterial cell surface and is thought to serve as a conduit for protein translocation from the bacterium into the plant host cell (Weber et al. 2005). The two open reading frames (ORFs) downstream of *hrpE* correspond to *hpaB* and *hpaE*. HpaB is an essential pathogenicity protein and acts as a general chaperone for T3S system-dependent secreted proteins, whereas the role of *hpaE* in pathogenicity is unclear (Büttner et al. 2004, 2006). HrpF, encoded by the only gene in the *hrpF* transcriptional unit, presumably inserts into the host plasma membrane as part of a channel-like protein complex, which is essential for the translocation of effector proteins across the plant plasma membrane (Büttner et al. 2002; Rossier et al. 2000).

The expression of the genes in the *X. campestris* pv. *vesicatoria* *hrp* region is regulated by environmental signals. During bacterial growth in complete media, the *hrp* regulon is expressed at a very low level. Gene expression, however, is induced in

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plant tissue or in specific minimal media that are probably mimicking in planta conditions (Schulte and Bonas 1992), and is controlled by two regulatory genes, *hrpG* and *hrpX*. HrpG is a member of the OmpR family of two-component response regulators and activates, in most cases via the AraC-type regulator HrpX, a large regulon, including the *hrp* operons and genes for type III effector proteins (Noel et al. 2001; Wengelnik and Bonas 1996; Wengelnik et al. 1999). A conserved DNA motif (TTCGC-N₁₅-TTCGC), the plant-inducible promoter (PIP) box, has been identified in several *hrp* promoters and, therefore, was proposed to act as a *cis*-regulatory element essential for HrpX-dependent regulation (Fenselau and Bonas 1995). Indeed, base substitutions in the PIP box resulted in a reduction of the HrpX-dependent gene expression in *X. oryzae* pv. *oryzae* and *X. campestris* pv. *vesicatoria* (Koebnik et al. 2006; Tsuge et al. 2005). Although PIP box promoters have been described upstream of the *hrpB*, *hrpC*, and *hrpD* transcriptional units, no PIP box-like element is present in a reasonable distance of the transcriptional start site of *hrpE*, although the expression is HrpX dependent (Weber et al. 2005; Wengelnik and Bonas 1996). Furthermore, no promoter activity could be identified in the region 1.2 kb upstream of *hrpE*, which encompasses *hrcD* and *hrpD6* (K. Wengelnik and U. Bonas, *unpublished*). These findings suggested that the *hrpE* promoter is situated further upstream and that *hrcD*, *hrpD6*, and *hrpE* may be located on the same transcriptional unit. However, in the initial complementation analyses based on Tn3-*gus* insertions, no insertions were obtained in *hrcD*, *hrpD6*, and *hrpE*, which made it difficult to allocate these genes to corresponding operons (Bonas et al. 1991).

Sequence analyses suggested the assignment of *hrcD* and *hrpD6* to the *hrpD* operon, because they appeared to have

overlapping translational start codons with the genes *hpaA* and *hrcD*, respectively, localized upstream. Because the end of the *hrpD6* coding sequence (CDS) and the start of the *hrpE* CDS are separated by 82 bp, *hrpE* was assigned with *hpaB* and *hpaE* to the *hrpE* operon.

Therefore, we set out to revisit the operon structure of the *hrpD* and *hrpE* region. Based on new complementation analyses, *hrcD*, *hrpD6*, and *hrpE* belong to the *hrpE* operon. In addition, the *hrpE* promoter was identified in *hpaA* and harbors a so far not considered PIP box that is conserved in all *Xanthomonas* strains.

RESULTS

Complementation analysis of the right region of the *hrp* gene cluster.

The promoter that drives expression of the Hrp pilus gene *hrpE* could not be identified in previous studies (see Materials and Methods). To revisit the organization of the transcription units present in the right part of the *hrp* gene cluster of *X. campestris* pv. *vesicatoria*, we performed complementation studies with nonpathogenic strains carrying transposon insertions in the promoter region upstream of *hrcQ* (strain 85-10::D140), in *hpaA* (strain 85-10::D29), and in *hpaB* (strain 85-10::E75), which were used in a previous study (Bonas et al. 1991) (Fig. 1A). In addition, we included transposon insertions in *hrcD* (strain 85-10::D506), *hrpD6* (strain 85-10::DGPS), and *hrpE* (strain 85-10::E84) (Table 1). The insertion sites were mapped by DNA sequencing or restriction enzyme analysis. For complementation analyses, cosmids carrying transposon insertions in the promoter region upstream of *hrcQ* (pD140), in *hrcD* (pD506), in *hrpE* (pE84), or in *hpaB* (pE75)

Fig. 1. Transcriptional organization and complementation analysis of the *hrpD* to *hrpE* region from *Xanthomonas campestris* pv. *vesicatoria*. **A**, New operon structure based on the complementation data presented in **B**. *hrcD*, *hrpD6*, and *hrpE* constitute the *hrpE* operon (filled arrows), which is located between the *hrpD* operon, containing *hrcQ*, *hrcR*, *hrcS*, and *hpaA* (open arrows), and *hpaB* (gray arrow). Open lollipops represent transposon insertions, small filled lollipops refer to predicted plant-inducible promoter boxes and the asterisk to the putative transcriptional start site upstream of the *hrpE* gene (Weber et al. 2005). Reverse-transcription polymerase chain reaction fragments spanning the intergenic regions are indicated by black lines. **B**, Complementation analysis. Cosmids carrying part of the *hrp* gene cluster with transposon insertions in the promoter upstream of *hrcQ* (pD140), in *hrcD* (pD506), in *hrpE* (pE84), and in *hpaB* (pE75) were conjugated into the indicated *X. campestris* pv. *vesicatoria* strains carrying chromosomal transposon insertions. The transconjugants were tested by inoculation of ECW-10R plants for the induction of the hypersensitive response (HR); + = HR induction and - = no HR induction.

were introduced into the chromosomal transposon mutants (Fig. 1B). From each experiment, two transconjugants were inoculated into leaves of pepper ECW-10R plants to test for their ability to elicit the HR similar to that which is induced by the wild-type strain 85-10. Pairs of transposon insertions that failed to complement each other in trans were considered to be located in the same complementation group. The results show that *hrcD*, *hrpD6*, and *hrpE* belong to the same operon, hereafter referred to as the *hrpE* operon. In contrast, *hpaB* belongs to a different operon (Fig. 1B).

Identification of transcripts in the *hrpD* and *hrpE* region.

The surprising fact that *hrpD6* and *hrpE*, separated by 82 bp, belong to the same operon prompted us to confirm the complementation data by reverse-transcription polymerase chain reaction (RT-PCR). Furthermore, we tested whether *hpaE*, the gene downstream of *hpaB*, is co-transcribed with *hpaB*. RT-PCR analyses were performed using cDNA derived from RNA isolated from strains 85-10 and 85* grown under *hrp* noninducing conditions. Strains 85-10 and its derivative 85* differ in their *hrp* gene expression status. Strain 85* expresses *hrpG**, a mutated form of the key regulatory gene *hrpG*, leading to constitutive expression of the *hrp* genes under normally noninducing conditions (Wengelnik et al. 1999). The oligonucleotides were designed in such a way that each primer pair anneals to two neighboring genes, thus creating a PCR product only when the gene pairs were co-transcribed (Fig. 1A). In the case of cDNA from strain 85*, PCR products spanning *hrcD*-*hrpD6* and *hrpD6*-*hrpE* were obtained, whereas no signals were detectable for *hpaA*-*hrcD*, *hrpE*-*hpaB*, and *hpaB*-*hpaE* (Fig. 2). As expected, no transcripts were detectable in

RNA from strain 85-10. These data indicate the presence of a transcript spanning the genes *hrcD*, *hrpD6*, and *hrpE* under *hrp* gene-inducing conditions and confirm the *hrpE* operon structure. The data also indicate that the *hpaB* and *hpaE* transcripts are derived from separate transcriptional units.

Mapping of the transcriptional start site of the *hrpE* operon.

From promoter studies with *hrp*::Tn3- β -glucuronidase (*gus*) fusions, it was known that the basal transcription level of the *hrpE* gene in complex medium is upregulated in XVM2 minimal medium and in planta (Wengelnik and Bonas 1996). Primer extension analysis defined a transcriptional start site of *hrpE*, but no regulatory sequences such as a PIP box or canonical sequence elements of σ 70-regulated promoters were identified (Weber et al. 2005). Based on the new *hrpE* operon structure, we scrutinized the *hrcD* region and found a perfect PIP box overlapping with the predicted translational start of *hrcD*. Interestingly, this PIP box is conserved between all six sequenced *Xanthomonas* strains. This finding prompted us to perform 5' RACE experiments to define the transcriptional start site of *hrcD*, the first gene in the newly defined *hrpE* operon (Fig. 3A). DNA sequencing of the RACE clones indicated that *hrpE* transcription starts at the adenosine 44 bp downstream of the PIP box, preceded by a consensus -10 region (Koebnik et al. 2006; Tsuge et al. 2005). As a consequence, the putative translational start codon of *hrcD*, downstream of a possible Shine-Dalgarno sequence, is located 91 codons downstream of the previously predicted translational start site, resulting in an HrcD protein consisting of 221 amino acids (24 kDa).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source ^b
<i>Xanthomonas campestris</i>		
pv. <i>vesicatoria</i>		
85-10	Pepper race 2; Rif ^r	Bonas et al. 1989
85*	85-10 Derivative carrying <i>hrpG*</i> , constitutive <i>hrp</i> gene expression	Wengelnik et al. 1999
85E*	<i>gumG</i> -Tn3- <i>gus</i> insertion mutant of 85*, <i>hrp</i> ⁺ , EPS ⁺ ; Km ^r	Wengelnik et al. 1996
85-10::D140	Tn3- <i>gus</i> insertion in the promoter of <i>hrpD</i>	Bonas et al. 1991
85-10::D29	Tn3- <i>gus</i> insertion in <i>hpaA</i>	Bonas et al. 1991
85-10::D506	Tn3- <i>gus</i> insertion in <i>hrcD</i>	Huguet et al. 1998
85-10::E75	Tn3- <i>gus</i> insertion in <i>hpaB</i>	Bonas et al. 1991
85-10::E84	Tn5 insertion in <i>hrpE</i>	Bonas et al. 1991
85-10::DGPS	Tn7 insertion in <i>hrpD6</i>	This study
<i>Escherichia coli</i>		
DH10b	F ⁻ <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX</i> 74 <i>endA1</i> <i>recA1</i> <i>deoR</i> <i>ara</i> Δ 139 Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ ⁻ <i>nupG</i> <i>rpsL</i> (Sm ^r)	Invitrogen
DH5 α λ _{pir}	F ⁺ ϕ 80 <i>dlacZ</i> Δ M15(λ _{pir}) Δ (<i>lacZYA</i> - <i>argF</i>)U169 <i>endA1</i> <i>recA1</i> <i>deoR</i> <i>hsdR17</i> (<i>rK</i> ⁻ <i>mK</i> ⁺) <i>phoA</i> <i>supE44</i> <i>thi-1</i> <i>gyrA96</i> <i>relA1</i> , Nal ^r ; host for propagation of pOK and pLAFR3 derivatives	Menard et al. 1993
Plasmids		
pOK1	Suicide vector; <i>sacB</i> <i>sacQ</i> <i>mobRK2</i> <i>oriR6K</i> ; Spc ^r	Huguet et al. 1998
pXV4	pLAFR3 clone from <i>X. campestris</i> pv. <i>vesicatoria</i> 75-3 genomic library, contains <i>hrcV</i> to <i>hrpF</i> ; Tc ^r	Wengelnik and Bonas 1996
pXV9	pLAFR3 clone from <i>X. campestris</i> pv. <i>vesicatoria</i> 75-3 genomic library, contains <i>hrcC</i> to <i>hpaE</i> ; Tc ^r	Bonas et al. 1991
pRK2013	Helper plasmid for triparental matings; <i>traRK</i> ⁺ <i>Mob</i> ⁺ ; Km ^r	Figurski and Helinski 1979
pCR2.1	Ap ^r , Km ^r	Invitrogen
pENTR/D-TOPO	Cloning vector; <i>attL</i> recombination sites; pUC- <i>ori</i> , Km ^r	Invitrogen
pL6GW356	pLAFR6 derivative containing Gateway (Invitrogen, Carlsbad, CA) <i>attR</i> reading frame B cassette; Cm ^r , Spc ^r	Noel et al. 2003
pGPS5	Transprimer donor; Km ^r , Ap ^r	New England Biolabs
pD140, pE75	pXV9 derivatives carrying Tn3- <i>gus</i> in the promoter of <i>hrpD</i> , <i>hpaA</i> or <i>hpaB</i> , respectively	Schulte and Bonas 1992
pE84	pXV9 derivative carrying Tn5 in <i>hrpE</i>	Bonas et al. 1991
pD506	pXV4 derivative carrying Tn3- <i>gus</i> in <i>hrcD</i>	Huguet et al. 1998
pOK- <i>hrpE</i>	pOK1 <i>hrcD</i> to <i>hpaE</i> in pOK1	Weber et al. 2005
pHrpE+PIP/-PIP	pLAFR6 expressing a translational fusion protein between HrpE and AvrBs3 Δ 2	This study

^a Rif^r, Km^r, Sm^r, Nal^r, Spc^r, Tc^r, and Cm^r = resistant to rifampicin, kanamycin, streptomycin, nalidixic acid, spectinomycin, tetracycline, and chloramphenicol, respectively.

^b Invitrogen: Carlsbad, CA, U.S.A. and New England Biolabs: Beverly, MA, U.S.A.

The PIP box promoter upstream of *hrcD* is responsible for HrpE expression.

To clarify whether or not the PIP box promoter upstream of *hrcD* might be responsible for the expression of the Hrp pilus subunit HrpE, or whether another internal promoter localized between *hrcD* and *hrpE* drives the expression of HrpE, a translational fusion between HrpE and the reporter protein AvrBs3Δ2 was constructed. AvrBs3Δ2, an N-terminal deletion derivative of the *X. campestris* pv. *vesicatoria* effector protein AvrBs3, serves as an established translational reporter in our group (Noel et al. 2003). The expression of the fusion protein was under the control of the 1.7- and 1.2-kb *hrpE* upstream sequence, differing in the presence of the PIP box in front of *hrcD* (pHrpE +PIP, pHrpE -PIP). The AvrBs3Δ2 fusion constructs were introduced into *X. campestris* pv. *vesicatoria* 85E*, which lacks the *avrBs3* gene. Western blot analysis of total protein extracts demonstrated that HrpE-AvrBs3Δ2 was expressed only under control of the 1.7-kb *hrpE* upstream sequence. No signal was detected in case of the 1.2-kb promoter fragment (Fig. 3B). This result clearly shows that the promoter, which controls the expression of the *hrpE* gene, is located upstream of *hrcD* and that there is no additional internal promoter.

Secondary structure of the intergenic *hrpD6*-*hrpE* region.

Based on the absence of a promoter immediately upstream of the *hrpE* gene, it appears unlikely that the mRNA start site in the region between *hrpD6* and *hrpE*, as revealed by previous primer extension experiments (Weber et al. 2005), represented a transcriptional start site. Interestingly, mRNA processing has been described for fimbrial gene clusters as part of a post-transcriptional regulatory mechanism (Balsalobre et al.

2003; Nilsson and Uhlin 1991). Because mRNA secondary structures often are involved in mRNA stabilization, we screened the intergenic region between *hrpD6* and *hrpE* (82 bp) for conserved mRNA secondary structures in the two most divergent *Xanthomonas* pathovars, *X. campestris* pvs. *campestris* and *vesicatoria*. Both share only 69% sequence identity in this region (Weber and Koebnik 2006). To find the lowest free energy structure common to the two RNA sequences, we applied the Dynalign algorithm. The advantage of this approach is the significant improvement in accuracy of structure prediction by finding shared structural elements (Mathews 2005). Dynalign revealed a conserved secondary structure formed by three stem loops (labeled I to III) (Fig. 4). In contrast to the conserved position and length of the stems, the loops, especially loops I and III, exhibited a higher sequence divergence. Interestingly, the previously identified mRNA start site is located in the single-stranded region immediately upstream of loop I and, therefore, may represent an mRNA-processing site. RNA processing would result in a transcript, which is stabilized by three conserved mRNA stem loops in its 5' untranslated region.

Fig. 2. Expression profiles of the *hpaA* to *hpaE* region. Neighboring genes were analysed by reverse-transcription polymerase chain reaction (RT-PCR) analysis of *Xanthomonas campestris* pv. *vesicatoria* strains 85-10 (85) and 85* grown in NYG using primers corresponding to adjacent genes. Genomic DNA and water serve as positive and negative control of the PCR, respectively. 16S ribosomal DNA was used as a standard (*rDNA*). RT-PCR samples were separated on a 1.5% agarose gel and stained with ethidium bromide.

A

B

Fig. 3. **A**, DNA sequence of the region between *hpaA* and *hrcD*. The sequence presented starts 24 bp upstream of the previously predicted *hrcD* translation start codon. The TGA stop codon of the preceding *hpaA* gene is underlined and the predicted plant-inducible promoter (PIP) box is shown in bold. An arrow indicates the transcriptional (+1) start site of the *hrpE* transcriptional unit, identified by 5'-RACE. The first possible translational start codon is boxed; SD (Shine-Dalgarno) refers to a putative ribosome binding site. The sequence of the deduced translation product is given in the single-letter code below the DNA sequence. **B**, Expression of the HrpE-AvrBs3Δ2 fusion protein. *X. campestris* pv. *vesicatoria* strain 85E* (wt) containing pHrpE -PIP or pHrpE +PIP was incubated in MA medium. Total protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% polyacrylamide) and analyzed by immunoblotting using an AvrBs3-specific antibody.

Genetic organization and evolution of the *hrpE* operon.

To investigate whether the genetic organization of the *hrpE* transcriptional unit is conserved in group II *hrp* gene clusters, we compared the predicted operon structures of *X. campestris* pv. *vesicatoria*, *R. solanacearum*, and *A. avenae* (Fig. 5A). Although the *hrpA* and *hrpB* operons show different organizations in the three species, the genes of the *hrpC* and *hrpD* operons are arranged in the same order and transcribed in the same direction in the three bacterial genera. The *hrpC* and *hrpD* operons encode the most conserved inner membrane components of the T3S system, *hrcU*, *hrcV*, *hrcQ*, *hrcR*, and *hrcS*, indicating that they represent the conserved core of the *hrp* gene cluster. Intriguingly, the conserved gene arrangement includes only the first four genes in the *hrpD* operon, up to *hpaA*. The only gene downstream of *hpaA* that is common to *X. campestris* pv. *vesicatoria*, *R. solanacearum*, and *A. avenae* is *hpaB*. Interestingly, in *A. avenae*, *hpaA* is followed by *hpaB*; whereas, in *X. campestris* pv. *vesicatoria*, the *hrpE* operon and, in *R. solanacearum*, four functionally related genes are present upstream of *hpaB*. This structural organization suggests that the additional genes were acquired later.

To test this hypothesis, we compared the synonymous (silent) substitutions per synonymous site (K_s) between the *hrpD* and the *hrpE* operon of *X. campestris* pv. *vesicatoria*. The K_s value commonly is regarded as a measure of the underlying mutational rate (Miyata et al. 1980). For reference, two housekeeping genes, malate dehydrogenase (*mdh*) and glyceraldehyde-3-phosphate dehydrogenase (*gapA*), were used (Weber and Koebnik 2006). Most notably, the K_s value of the *hrpE* operon was elevated compared with the housekeeping genes and the genes in the *hrpD* operon, indicating a higher mutational rate for the *hrpE* operon (Fig. 5B). From these results, we conclude that the *hrpE* operon may represent a functional unit which was affiliated to the core *hrp* gene cluster at a later time.

DISCUSSION

In this study, we defined a new operon structure in the *hrpD* to *hrpE* region of the *hrp* gene cluster from *X. campestris* pv. *vesicatoria*. In contrast to previous predictions, the *hrpD* transcriptional unit encompasses *hrcQ*, *hrcR*, *hrcS*, and *hpaA*, whereas the *hrpE* operon contains *hrcD*, *hrpD6*, and *hrpE*. The *hrpE* operon is flanked on the right side by the monocistronic operons *hpaB* and *hpaE* (Fig. 1A). Nonpolar deletion mutants in each gene of the *hrpE* operon had a clear Hrp-negative phenotype (i.e., they were unable to cause disease in susceptible pepper plants and to induce the HR in resistant pepper plants) (Huguet et al. 1998; Weber et al. 2005).

The only protein encoded by the *hrpE* operon characterized in detail is HrpE, the major subunit of the Hrp pilus (Weber et al. 2005). HrpE, which is unique for the genus *Xanthomonas*, possesses a two-domain structure with a surface-exposed, positively selected N terminus and a C-terminal polymerization domain (Weber and Koebnik 2005, 2006). *hrpD6* is a small and unique gene in the *hrp* gene cluster of *Xanthomonas*. A mutation in the synthetic gene in *R. solanacearum*, *hrpX*, resulted in a loss of Hrp pili but still allowed the transport of the Hrp pilus subunit HrpY (Van Gijsegem et al. 2002). The first ORF in the *hrpE* operon encodes HrcD, a bitopic membrane protein that exhibits a weak homology to the members of the YscD family (Pallen et al. 2005; C. Berger and R. Koebnik, unpublished). Other members of this protein family are HrpW from *R. solanacearum* and PrgH from *Salmonella typhimurium*. PrgH is an essential component of the T3S needle complex associated with the T3S system and has been copurified with the needle subunit PrgI (Kubori et al. 1998). The extracellular needle of human pathogens corresponds in func-

tion to the Hrp pili of plant-pathogenic bacteria. From these observations, we conclude that the three genes of the *hrpE* operon form a functional unit involved in a more specialized role in Hrp pilus assembly than the rest of the *hrp* gene cluster, which most probably encodes the core components of the T3S system.

Sequence comparisons between the *X. campestris* pv. *vesicatoria*, *R. solanacearum*, and *A. avenae* *hrp* gene clusters revealed that the *hrpC* and *hrpD* operons exhibit an identical gene arrangement. This conserved region is followed by the *hrpE* operon in *Xanthomonas* spp. Intriguingly, genes corresponding to components of the *hrpE* operon in terms of gene synteny and homology are present in *R. solanacearum* but are missing in *A. avenae*. The elevated mutational rate, as indicated by the K_s values of the *hrpE* operon, led us to speculate that this region was acquired later than the core *hrp* gene cluster. The increased mutational rate might be an indication of a "species-adaptive" process in which the acquired DNA gradually becomes indistinguishable from the rest of the genome in terms of G+C content and codon usage. We do not know if a pilus-specific gene cluster is present elsewhere in the *A. avenae* genome.

Fig. 4. Predicted secondary structures of the *hrpD6*-*hrpE* intergenic region. The 120 bp upstream of the *hrpE* translational start codon of **A**, *Xanthomonas campestris* pv. *vesicatoria* 85-10 and **B**, *X. campestris* pv. *campestris* ATTC33913 were analyzed using the Dynalign algorithm, as implemented in the RNAstructure program (Mathews 2005). ΔG_0 value (Gibbs free energy) for both sequences is -49.1 kcal/mol. The putative mRNA processing site identified in *X. campestris* pv. *vesicatoria* is encircled. The stop codon of *hrpD6* and the translational start codon of *hrpE* are shown in bold.

The expression data presented here confirm that the *hrpE* operon is upregulated under *hrp*-inducing conditions. We identified a promoter upstream of the *hrcD* gene that harbors a canonical PIP box 44 bp upstream of the transcriptional start site of the *hrpE* operon. A similar spacing of 45 ± 2 bp between the last conserved guanine residue of the second PIP half-box and the mapped transcriptional start sites, as well as sequence and position of the -10 region, are in concordance with the functionally characterized PIP box promoters (Fenselau and Bonas 1995; Koebnik et al. 2006; Tsuge et al. 2005). One reason why this PIP box promoter was not considered previously was its localization in the *hrcD* coding region. The transla-

tional start codon of *hrcD*, redefined in this study, does not affect the predicted HrcD transmembrane helix. The expression of a C-terminally hexahistidine-tagged HrcD in *X. campestris* pv. *vesicatoria* led to the purification of a 25-kDa protein, which corresponds to the expected size of HrcD starting at the suggested start codon. However, due to N-terminal modification of the protein, the N-terminal amino acid sequence could not be determined (C. Berger and R. Koebnik, *unpublished*).

In addition to PIP boxes, other mechanisms may be important for the regulation of *hrp* gene expression (e.g., RNA processing). By comparisons with *X. campestris* pv. *campestris*, a more distantly related *Xanthomonas* sp., we predicted con-

Fig. 5. *hrpE* transcriptional unit is suggested to have been acquired later to the *hrp* gene cluster. **A**, Genetic organization of group II *hrp* gene clusters in *Xanthomonas campestris* pv. *vesicatoria*, *Ralstonia solanacearum*, and *Acidovorax avenae* subsp. *citrulli*. Transcriptional units are indicated by a black line. Conserved *hrc* genes are represented by gray arrows; *hrp* genes by open white arrows, *hpa* genes by filled black arrows, putative type III effectors by dotted black arrows, and regulators by hatched white arrows (for *A. avenae* based on homology only). Plant-inducible promoter boxes are indicated by black circles. The conserved core of the *hrp* gene clusters is boxed. **B**, Comparison of the synonymous (silent) substitutions per synonymous site (K_s) values between genes encoding the malate dehydrogenase (*mdh*), glyceraldehyde-3-phosphate dehydrogenase (*gapA*), *hrpD*, and *hrpE* operon. *X. axonopodis* pv. *glycines* strain 8ra was not included in the analysis of *mdh* and *gapA* due to lack of sequence information.

served stem loops in the mRNA located upstream of the *hrpE* gene. These secondary structures could be involved in the posttranscriptional regulation of the *hrpE* operon. Such a regulation would allow differential expression of individual genes in the polycistronic *hrpE* operon, because the Hrp pilus subunit HrpE and the T3S system components HrpD6 and HrcD certainly are required in nonstoichiometric amounts. A well-studied example of how differential expression of individual genes in polycistronic transcripts can be achieved is the *pap* gene cluster of uropathogenic *Escherichia coli* strains encoding pyelonephritis-associated pili (Nilsson and Uhlin 1991). In this case, the mRNA is subjected to several processing events, resulting in a variety of transcripts with different stabilities. The most stable transcript generated during RNase E-dependent processing corresponds to the *papA* transcript which encodes the major fimbrial component PapA. Interestingly, the *papA* transcript includes a stem loop located at the 5' end which stabilizes the transcript by protecting it from degradation (Bricker and Belasco 1999). Based on these data, we assume that the previously identified transcriptional start site of the *hrpE* gene is, in fact, a processing site and mRNA processing might result in a *hrpE* transcript that is stabilized by stem loop structures at its 5' end. In accordance with these speculations, the *hrpA* transcript, which encodes the Hrp pilus subunit HrpA of *P. syringae*, shows an unusually long mRNA half-life time. Furthermore, the 5' nontranslated region of *hrpA* strongly increases the stability of the *hrpA* transcript and the amount of HrpA protein (Hienonen et al. 2002, 2004). To gain further insight into the potential parallel between the regulatory principles of fimbrial gene clusters and *hrp* gene clusters, it will be interesting to determine the biological significance of the predicted stem-loop structures in the transcript of the Hrp pilus protein and to address the mechanism of the probable mRNA processing.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used are described in Table 1. Plasmids were introduced into *E. coli* by electroporation and into *X. campestris* pv. *vesicatoria* by conjugation with pRK2013 as a helper plasmid in triparental matings (Ditta et al. 1980). *E. coli* cells were cultivated at 37°C in Luria-Bertani medium, and *X. campestris* pv. *vesicatoria* strains were cultivated at 30°C in NYG (Daniels et al. 1984), or in MA minimal medium (Rossier et al. 1999). Antibiotics were added to the media at the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 25 µg/ml; tetracycline, 10 µg/ml; rifampicin, 100 µg/ml; and spectinomycin, 100 µg/ml.

Plant material and plant inoculations.

Pepper cultivar ECW-10R is a near-isogenic line of ECW carrying the resistance gene *BsI* (Minsavage et al. 1990). Inoculation of plant leaves was performed as described previously (Bonas et al. 1991).

Generation of transposon insertion mutants.

The GPS-LS mutagenesis kit was used to create a transposon insertion mutant in *hrpD6* according to the manufacturer's instructions (New England Biolabs, Beverly, MA, U.S.A.). Briefly, the mobilizable suicide plasmid pOK-*hrpE* was used as the target DNA for mutagenesis (Weber et al. 2005). *E. coli* DH5α λ_{pir} transformants were selected for spectinomycin and kanamycin resistance. Insertions in *hrpD6* were mapped by colony PCR using a transposon-specific primer supplied by the manufacturer and the plasmid-specific primer hrpE(-200) (5'-GCA TGA GCT CGA AAT CCC AAG CAC ATG ACA TCC

CTG C). To determine the exact insertion site, positive clones were sequenced. The wild-type copy of *hrpD6* in *X. campestris* pv. *vesicatoria* then was replaced by transposon insertion derivatives as described previously (Kaniga et al. 1991). The replacement, generated by two consecutive crossover events, was confirmed by PCR analysis.

Construction of HrpE-AvrBs3Δ2 fusion proteins and immunoblot analyses.

To create protein fusions of HrpE with the reporter protein AvrBs3Δ2, plasmid pL6GW356 (Noel et al. 2003), containing the Gateway *attR* reading frame B cassette (Invitrogen, Carlsbad, CA, U.S.A.) in front of *avrBs3Δ2*, was used. Fragments containing a 1.7- or 1.2-kb sequence upstream of the translational start of *hrpE* as well as the first 150 bp of the *hrpE* CDS were amplified from genomic DNA of *X. campestris* pv. *vesicatoria* 85-10 and cloned into pENTR/D-TOPO (Invitrogen). Primer sequences are available upon request. Recombination into pL6GW356 created the expression plasmids pHrpE +PIP and pHrpE -PIP. To analyze the expression of the HrpE-AvrBs3Δ2 fusion proteins, *X. campestris* pv. *vesicatoria* strains were grown at 30°C for 16 h in MA medium. Total protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Western blotting. Western blots were incubated with an AvrBs3-specific polyclonal antiserum (diluted 1:1,000) (Knoop et al. 1991) and with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Uppsala, Sweden), which was detected by enhanced chemiluminescence (Amersham Biosciences).

RT-PCR analyses.

Bacteria were harvested by centrifugation at an optical density at 600 nm of 0.6. RNA was extracted by the hot phenol procedure (Aiba et al. 1981). RNA (5 µg) and 150 ng of random oligonucleotide hexamers were used to generate cDNAs using the TimeSaver cDNA Synthesis kit (Amersham Biosciences). RT-PCR analysis was performed with diluted cDNA (1:100) as template and 34 PCR amplification cycles. 16S rDNA served as a constitutive control. Sequences of the primers spanning *hpaA* and *hrcD*, *hrcD* and *hrpD6*, *hrpD6* and *hrpE*, *hrpE*, and *hpaB* and *hpaB* and *hpaE* are available on request.

Mapping of the *hrcD* transcriptional start site.

Total RNA was extracted from *X. campestris* pv. *vesicatoria* 85E* grown for 16 h in NYG using the Qiagen RNeasy mini kit (Qiagen GmbH, Hilden, Germany). 5'-RACE was performed with the SMART RACE cDNA Amplification kit (Clontech Laboratories, Mountain View, CA, U.S.A.) following the manufacturer's instructions. First-strand synthesis was carried out with the gene-specific primer *hrpD5*-256 (5'-CAG CAG CAT CGC CAA CGC ATA GCA C), annealing 256 bp downstream of the proposed translational start, and the SMART II oligo (5'-AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG). The cDNA was amplified in a second PCR using an adapter-specific forward primer, UPM (5'-CTA ATA CGA CTC ACT ATA GGG C), and gene-specific reverse primer *hrpD5*-186 (5'-TTG CAC CGT GTC GCT G). 5'-RACE products were cloned into pCR2.1 (Invitrogen) and sequenced.

Bioinformatic analyses.

To predict secondary structures, the Dynalign algorithm, as implemented in the RNAstructure program (Mathews 2005), version 4.2, was applied using default parameters. The calculations of synonymous substitutions per synonymous site (*K_s*) were performed with the MEGA 2 software (Kumar et al.

2001), version 2.1, using the modified Nei-Gojobori method with the Jukes-Cantor corrected distance and the complete-deletion-of-gaps option. The sequences of single CDS from *X. campestris* pv. *vesicatoria* 85-10 (GenBank accession number [AC] AM039948), *X. oryzae* pv. *oryzae* KACC 10331 (AC AE013598), *X. axonopodis* pv. *glycines* 8ra (AC AF499777), *X. axonopodis* pv. *citri* 306 (AC AE011665), and *X. campestris* pv. *campestris* ATCC 33913 (AC AE012221) were aligned using CLUSTAL X and combined to one contiguous sequence prior to the analyses (Thompson et al. 1997).

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